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Increased amount of bone marrow-derived smooth muscle-like cells and accelerated atherosclerosis in diabetic apoE-deficient mice

J.O. Fledderus [a,1], O. van Oostrom [a,1], D.P.V. de Kleijn [b,c], K. den Ouden [a,b], A.F. Penders [a], H. Gremmels [a], P. de Bree [a], M.C. Verhaar [a,*]

[a] Laboratory of Renal and Vascular Biology, Department of Nephrology and Hypertension, F03.227, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
[b] Department of Experimental Cardiology, G02.523, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
[c] Interuniversity Cardiology Institute, Postbus 19258, 3501 DG Utrecht, The Netherlands

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ABSTRACT

Aims: Atherosclerotic plaque development is accelerated in patients with diabetes. Bone marrow-derived smooth muscle-like cells have been detected in neointima and diabetes has a numerical and functional effect on circulating vascular progenitor cells. We hypothesized that an increased number of bone marrow-derived smooth muscle-like cells correlates with accelerated atherosclerosis in diabetic apoE-deficient mice.

Methods: ApoE$^{-/-}$ mice were subjected to total body irradiation and transplanted with bone marrow cells from GFP-transgenic mice. Mice were rendered diabetic by streptozotocin injection and examined after 4, 8, 11 and 15 weeks of diabetes.

Results: Diabetic mice showed a larger plaque area and a higher number of smooth muscle-like cells compared to non-diabetic mice at 11 and 15 weeks after diabetes induction. Bone marrow-derived smooth muscle-like cells were detected in atherosclerotic plaques of both diabetic and control mice, but numbers were higher in plaques of diabetic mice 11 weeks after induction of diabetes. The higher number of bone marrow-derived smooth muscle-like cells in plaque was associated with an increase in *in vitro* differentiation of smooth muscle-like cells from spleen mononuclear cells in diabetic mice.

Conclusions: Diabetes increases the number of bone marrow-derived smooth muscle-like cells in atherosclerotic plaques and the differentiation of mononuclear cells towards smooth muscle-like cells, which may contribute to accelerated atherosclerotic plaque development in diabetic apoE$^{-/-}$ mice.

1. Introduction

Patients with diabetes are at a higher risk for developing atherosclerosis than non-diabetic subjects and have enhanced susceptibility for myocardial infarction, peripheral artery disease and stroke [1]. Hyperglycemia, dyslipidemia and hypertension only partly explain this increased incidence of macrovascular complications in diabetes [2]. Diabetes induces phenotypic changes in vascular smooth muscle cells (SMC) such as increased contractility, cellular proliferation, migration and extracellular matrix formation [3], which may lead to accelerated SMC accumulation in atherosclerotic lesions [4]. The cellular and molecular mechanisms by which diabetes accelerates atherosclerotic cardiovascular disease are still poorly understood.

It has been hypothesized that bone marrow (BM)-derived smooth muscle-like cells (SMLC) are involved in neointima formation in various animal models of vascular diseases such as postangioplasty restenosis, transplant-associated atherosclerosis, and hyperlipidemia-induced atherosclerosis [5–8]. Whether or not these BM-derived cells differentiate into vascular SMC has been scrutinized in recent years [9]. An accumulating body of evidence from genetic lineage tracing in several models of arterial injury (mechanical injury, graft vasculopathy and chronic atherosclerosis) now suggests that BM-derived SMLC are of monocyte/macrophage lineage and may contribute to vascular remodeling and atherosclerosis in a paracrine fashion, but that it is unlikely that they differentiate into a definitive smooth muscle cell lineage [10–14].

Interestingly, diabetes has a numerical and functional effect on circulating vascular progenitor cells [15,16]. In human subjects,

* Corresponding author. Tel.: +31 88 755 7329; fax: +31 30 251 8328.
E-mail address: m.c.verhaar@umcutrecht.nl (M.C. Verhaar).
[1] Contributed equally.

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SMLC have been obtained from peripheral blood. Prior to differentiation, these cells could be characterized by positive staining for CD14, CD105, and α-smooth muscle actin (α-SMA) and during *in vitro* culture they developed additional SMC markers including SM22α and calponin, but not smooth muscle myosin heavy chain (SM-MHC) [17]. In another study, it was shown that peripheral blood mononuclear cells (MNC) differentiated into PDGFR-β expressing SMLC after stimulation with TGF-β *in vitro* [18]. In type 2 diabetic patients, van Ark et al. observed a relative increase in circulating ($CD14^+CD105^+$) and cultured SMLC numbers compared to endothelial progenitor cells [19]. We recently reported that patients with type 1 diabetes have increased levels of collagen type I- and α-smooth muscle actin (α-SMA)-positive SMLC/myofibroblast progenitor cells after differentiation of peripheral blood MNC *in vitro* [20]. In addition, we showed that SMLC differentiation was accelerated and numeric outgrowth increased in diabetic mice [21].

The present study examined the role of BM-derived SMLC in accelerated atherosclerosis in diabetic mice. We generated chimeric mice by BM transplantation from green fluorescent protein (GFP)-transgenic mice into apoE-deficient mice ($GFP_{BM} \rightarrow apoE^{-/-}$) and subsequently injected these mice with vehicle (control animals) or streptozotocin (STZ) to induce diabetes. We hypothesized that an increased number of BM-derived SMLC is present in diabetic atherosclerotic plaques, which may correlate with accelerated plaque growth in diabetic $GFP_{BM} \rightarrow apoE^{-/-}$ mice.

2. Methods

A detailed description of the methods can be found in the Supplemental material.

2.1. Animals and bone marrow transplantation

Male C57BL/6 $apoE^{-/-}$ ($n = 63$) and C57BL/6 GFP-transgenic mice (strain name: C57Bl/6-Tg(UBC-GFP)30Scha/J; stock number: 004353) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). $ApoE^{-/-}$ mice were lethally irradiated (700cGy) and rescued by intravenous tail injection of 5×10^6 BM cells harvested from femurs of age- and sex-matched GFP^+ mice ($n = 20$). Normal chow and water were given *ad libitum*. All procedures were approved by the local ethical committee.

2.2. Induction of diabetes

At age 10–12 weeks, chimeric $GFP_{BM} \rightarrow apoE^{-/-}$ mice were randomly assigned to either the diabetic or non-diabetic control group. Mice in the diabetic group were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ) at a dose of 200 mg/kg (Serva, Germany) in citrate buffer. Control mice received citrate buffer alone. Non-fasting blood glucose levels were measured within a week after STZ injection and from then on every 4 weeks. Diabetic and control $GFP_{BM} \rightarrow apoE^{-/-}$ mice were sacrificed 4, 8, 11, and 15 weeks after induction of diabetes.

2.3. Blood sampling and tissue processing

After 4, 8, 11 or 15 weeks mice were anaesthetized by an intraperitoneal injection (10 μl/g body weight) of ketamine (6 mg/ml) and medetomidine (50 μg/ml). Blood samples were obtained by cardiac puncture. Perfusion of the arterial system was performed by cardiac puncture with 0.9% NaCl, after which the aorta was harvested and snap-frozen in liquid nitrogen and stored at −80 °C. The heart was stored overnight at 4 °C in a solution containing 0.2% paraformaldehyde and 4% sucrose to preserve the GFP fluorescence signal. This was followed by placing the heart in a 15% sucrose solution before being embedded in TissueTek OCT (Sakura) and frozen at −80 °C for histological analysis. The spleen was harvested and used directly for cell culture studies.

2.4. Biochemical measurements

Blood glucose levels were measured using a portable glucose meter (Medisense Precision Xtra; Abbott Laboratories, USA). Glycated hemoglobin was measured in peripheral blood samples by high-performance liquid chromatography. Total serum cholesterol levels were measured according to manufacturer's instructions (Elitech CHOL-0420 kit, Bio-Rad). Stromal cell-derived factor (SDF)-1α ELISA (R&D Systems) on plasma samples was performed according to manufacturer's instructions.

2.5. Immunohistochemical analysis

Serial 7 μm thick cross sections of the aortic sinus were stained with Oil Red O and counterstained with hematoxylin-eosin to measure atherosclerotic lesion area by image analyzing software (Soft Imaging Systems, Germany). Stainings were examined with light microscopy on the Olympus BX60 microscope by two observers blinded to the identity of the samples.

2.6. Immunofluorescence analysis

Double immunofluorescence staining with antibodies directed against α-GFP (Abcam), α-SMA (Sigma–Aldrich), caldesmon (Santa Cruz) and calponin (Sigma–Aldrich) was performed using cross sections of the aortic sinus. The nuclei were counterstained with DAPI (Invitrogen). Single or double-positive cells were defined as nucleated cells staining positive for GFP (green), α-SMA, caldesmon or calponin (red) or co-localization of GFP with one of the SMC markers (yellow). The presence of double-positive cells was confirmed in a subset of the sections by *z*-axis images on the confocal laser scanning microscope (LSM510). Macrophages were, stained with rat-monoclonal MOMA-2 antibody (Millipore).

2.7. Smooth muscle-like cell culture and flow cytometry

MNC were isolated from the spleen of $apoE^{-/-}$ mice or diabetic and control $GFP_{BM} \rightarrow apoE^{-/-}$ mice and 5×10^6 MNC/well were plated on fibronectin-coated dishes in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 20% heat-inactivated fetal bovine serum, antibiotics (penicillin 100U/ml and streptomycin 100 μg/ml Invitrogen). When indicated, platelet-derived growth factor (PDGF)-BB (5 ng/ml; R&D systems), transforming growth factor (TGF)-β (5 ng/ml; R&D systems), or both were added to the DMEM medium. After 3 or 7 days in culture, adherent cells were washed with PBS and detached using trypsin-EDTA and a cell scraper. SMLC were permeabilized, stained with α-SMA-FITC (R&D Systems) and counted in a flow cytometer (Beckman Coulter).

2.8. Real-time polymerase chain reaction

Real-time PCR was performed on cDNA from freshly isolated spleen MNC and spleen-derived SMLC cultured for 4 or 8 days using primers for α-SMA (mmActa2), calponin (mmCnn1), caldesmon (mmCald1) and SM-MHC (mmMyh11). The expression values of the genes of interest were normalized using the geometric average of the expression values of 3 housekeeping genes, beta-actin (mmActb), GAPDH (mmGapdh) and large ribosomal protein P0 (mmRplp0), and expressed as fold change relative to the spleen MNC sample for each gene. Primer sequences are listed in Supplemental Table I.

2.9. Statistical analysis

Data were analyzed in Sigmaplot by Student's *t*-test or ANOVA, and Student-Newman-Keuls method was used for comparisons of group means. Logarithmic transformation was performed on the number of cells in atherosclerotic plaques and on the number of SMLC after culturing because of non-normal distribution of the data. Data are expressed as mean ± SEM. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Characterization of the diabetic $GFP_{BM} \rightarrow apoE^{-/-}$ mouse model

3.1.1. BM transplantation

The degree of BM chimerism obtained by transplantation of GFP^+ BM to irradiated $apoE^{-/-}$ mice was assessed by flow cytometry of peripheral blood leukocytes. The fraction of GFP^+ peripheral blood leukocytes in $GFP_{BM} \rightarrow apoE^{-/-}$ mice 2 weeks after BM transplantation was 89 ± 5%. At the time of sacrifice this was 90 ± 3% in control $GFP_{BM} \rightarrow apoE^{-/-}$ mice and 86 ± 6% in diabetic $GFP_{BM} \rightarrow apoE^{-/-}$ mice, indicating successful long term replacement of the hematopoietic stem cell population.

3.1.2. Effect of diabetes on metabolic parameters in $GFP_{BM} \rightarrow apoE^{-/-}$ mice

On a chow diet, a reduction in body weight was observed in diabetic $GFP_{BM} \rightarrow apoE^{-/-}$ mice at 4, 8, 11 and 15 weeks after diabetes induction compared to control $GFP_{BM} \rightarrow apoE^{-/-}$ mice. Plasma glucose and glycated hemoglobin (HbA1C) were significantly higher in the diabetic mice compared to control mice at all time points confirming persistent hyperglycemia after STZ induction. Furthermore, the induction of diabetes was associated with higher total cholesterol levels after 4 and 11 weeks compared to control mice (Supplemental Table II).

3.2. Diabetic $GFP_{BM} \rightarrow apoE^{-/-}$ mice show accelerated atherosclerosis

Induction of diabetes resulted in accelerated development and progression of atherosclerosis. Larger plaques with a thickened intima composed of a fibrous cap and lipid-rich core were observed in diabetic $GFP_{BM} \rightarrow apoE^{-/-}$ mice (Fig. 1A–B). Oil Red O staining was performed for the assessment of atherosclerotic lesion area at the aortic sinus. In control $GFP_{BM} \rightarrow apoE^{-/-}$ mice, we detected typical fibrous fatty streaks which significantly increased in size over time (Fig. 1C). Quantitative analysis of atherosclerotic lesions showed an increase in plaque area over time. Furthermore, a significantly larger plaque area was observed in diabetic mice compared to control mice at 8, 11 and 15 weeks after diabetes induction (Fig. 1C).

Immunofluorescence staining for α-SMA was performed on atherosclerotic lesions (Fig. 2A–B). α-SMA^+ cells were present in plaques of diabetic and control animals and localized predominantly in the media, the fibrous cap and at the shoulders of the atherosclerotic plaque. Quantitative assessment of the number of α-SMA^+ cells in the plaque showed significant increase over time in diabetic mice and not in control mice (Fig. 2C). The number of α-SMA^+ cells was higher in diabetic plaques at 11 and 15 weeks. Comparing plaques of similar size from diabetic mice at 8 weeks and control mice at 15 weeks showed significantly more α-SMA^+ cells in the diabetic plaques.

3.3. GFP^+ cells are present in neointima of atherosclerotic lesions

GFP^+ cells were observed in plaques of control $GFP_{BM} \rightarrow apoE^{-/-}$ mice as well as in diabetic $GFP_{BM} \rightarrow apoE^{-/-}$ mice at all time points.

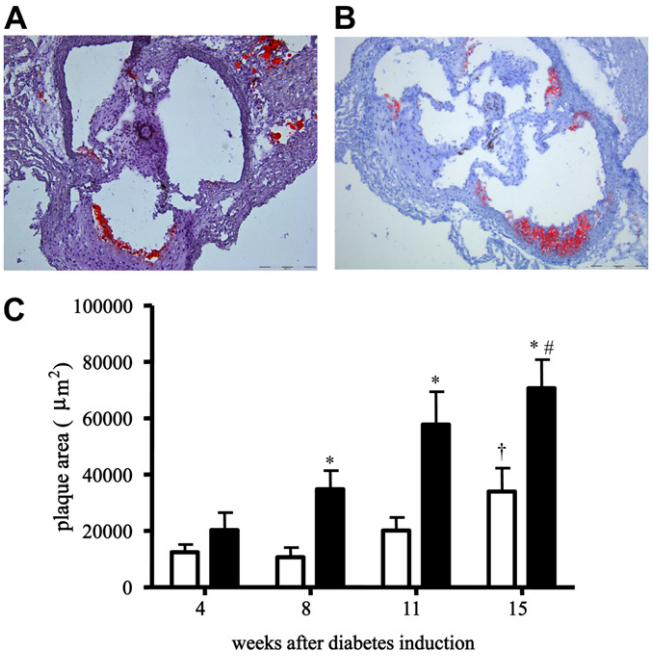

Fig. 1. Representative examples of oil red staining in aortic sinus from $apoE^{-/-}$ mice after 4 weeks (A) and after 15 weeks of diabetes (B), magnification ×40. Quantification of lesion size in control (white bar) and diabetic (black bar) mice (C). $^*p < 0.05$ versus control (paired time point); $\#p < 0.05$ versus 4 weeks.

Most GFP^+ cells were localized in the neointima and none were detected in the media (Fig. 3A). Over time, the number of BM cells did not increase in plaques of control mice. In diabetic plaques there was an increase in GFP^+ cells at 15 weeks compared to 4 weeks. Diabetic plaques contained higher numbers of GFP^+ cells compared to control plaques at 8, 11 and 15 weeks (Fig. 3B). In

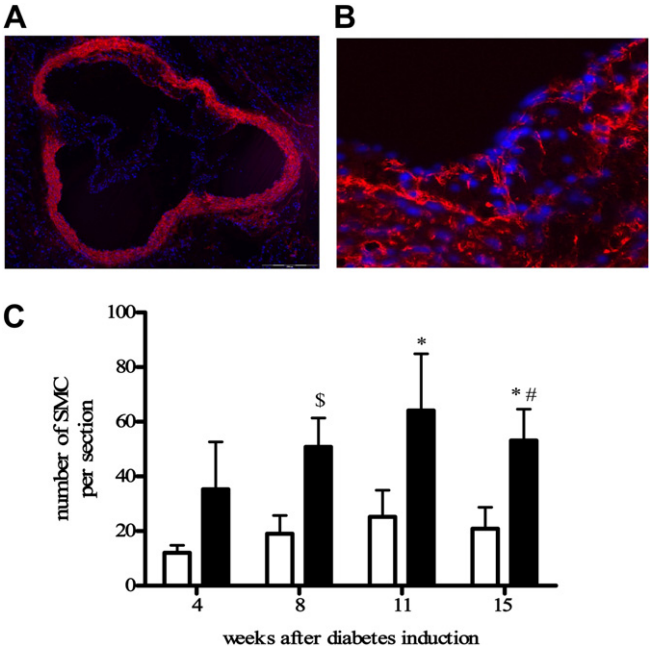

Fig. 2. Examples of α-SMA (red) staining. Cell nuclei are blue (DAPI) (A, B). SMC quantification (C). Control mice, white bar; diabetic mice, black bar. Magnification ×40, inset ×400. $^*p < 0.05$ versus control (paired time point); $\#p < 0.05$ versus 4 weeks; $\$p < 0.05$ versus 15 week controls.

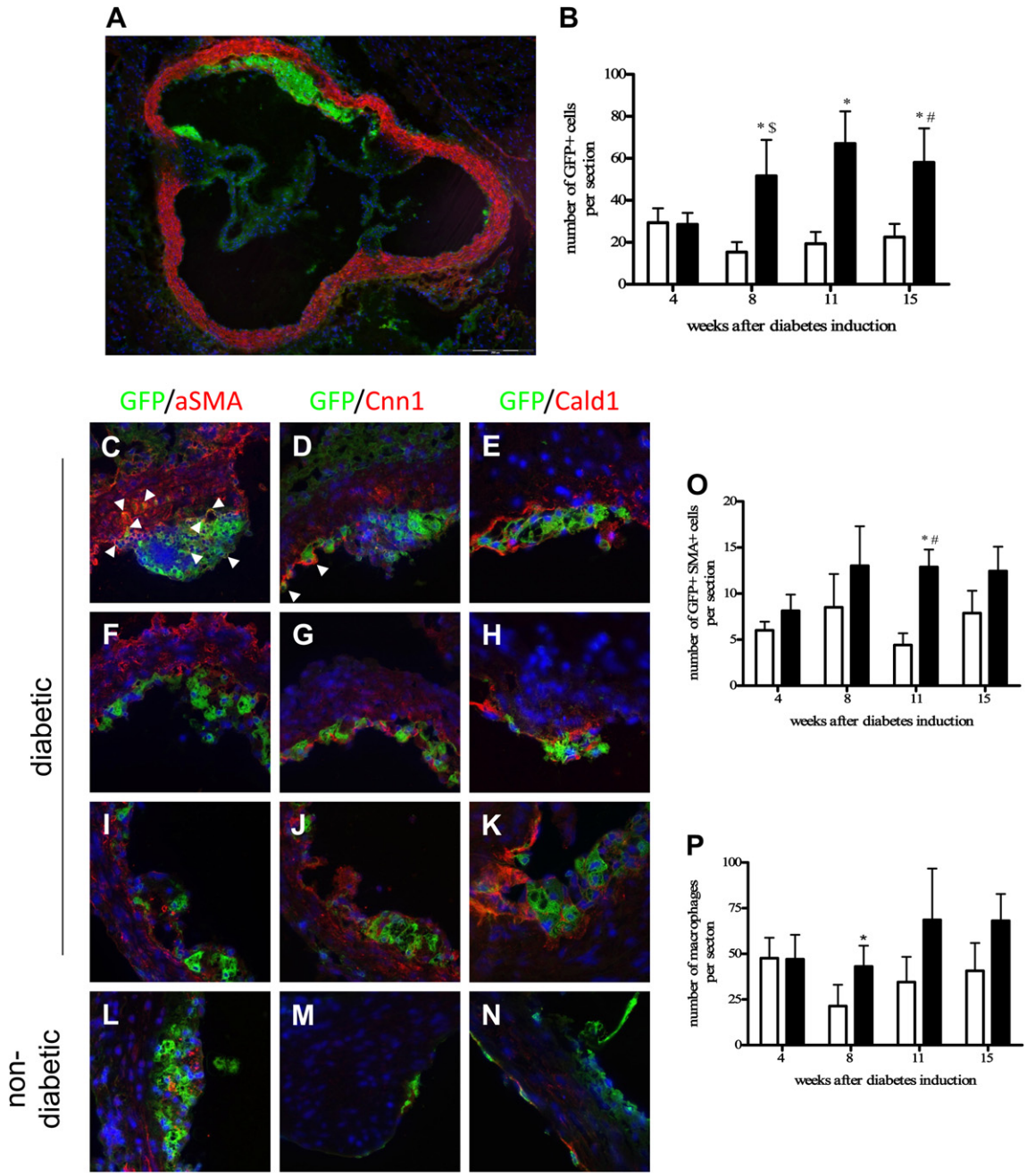

Fig. 3. BM-derived cell quantification. (A) Immunofluorescent triple staining of aortic root section for GFP (green), α-SMA (red) and cell nuclei (blue, DAPI). (B) Quantification of GFP-positive cells in the plaques. Control mice, white bar; diabetic mice, black bar, magnification ×40. (C–N) Representative immunofluorescent stainings on aortic root sections from diabetic or non-diabetic mice for α-SMA, Cnn1 (Calponin) or Cald1 (Caldesmon) (all red) co-stained with GFP (green) and cell nuclei (blue, DAPI). (O) Quantification of α-SMA/GFP double-positive cells and number of macrophages (P) in the plaques. Control mice, white bar; diabetic mice, black bar, magnification ×40. $^{*}p < 0.05$ versus control (paired time point); $\#p < 0.05$ versus 4 weeks; $\$p < 0.05$ versus 15 week controls.

addition, diabetic plaques at 8 weeks contained significantly more GFP^{+} cells compared to similar sized control plaques at 15 weeks.

3.4. $GFP^{+}/\alpha\text{-}SMA^{+}$ and $GFP^{+}/Cnn1^{+}$ double-positive cells are localized predominantly in the neointima

Double-staining for GFP with α-SMA, calponin or caldesmon showed that only a few round, nucleated GFP^{+} cells in atherosclerotic lesions was positive for α-SMA or calponin, but not caldesmon (Fig. 3C,D and Supp Fig. IJ,N). Cells that were double-positive for GFP and α-SMA/calponin were localized predominantly in the neointima or on the luminal side of the vessel wall. In the majority of plaques in diabetic and non-diabetic animals, however, no evidence of double-positive cells could be found (Fig. 3E–N). The observation that cells co-expressing GFP and α-SMA/calponin were topologically and morphologically distinct from medial SMC, suggests that they are not differentiated SMC but more closely resemble the profile of the previously described SMLC. We confirmed the presence of double-positive cells by using confocal microscopy (for movies of *z*-stack reconstructed

images see Supplemental material), in accordance with recent literature [9].

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.atherosclerosis.2012.11.017.

3.5. Diabetes increases the amount of GFP$^+$ α-SMA$^+$ cells in atherosclerotic lesions

We quantified GFP$^+$ α-SMA$^+$ cells in atherosclerotic plaques of diabetic and control GFP$_{BM}$→apoE$^{-/-}$ mice (Fig. 3O). In control mice, we found no significant differences over time in the number of plaque GFP$^+$ α-SMA$^+$ cells. Diabetic plaques showed an increased amount of GFP$^+$ α-SMA$^+$ cells at 11 compared to 4 weeks after diabetes induction. In addition, the number of GFP$^+$ α-SMA$^+$ cells was higher in diabetic versus control plaques at 11 weeks, and similar sized plaques from diabetic mice at 8 weeks and control mice at 15 weeks showed a trend towards higher numbers of GFP$^+$ α-SMA$^+$ cells in the diabetic mice. As only a part of the BM cells could be characterized as α-SMA$^+$ cells, we also determined the number of macrophages in atherosclerotic plaques of control and diabetic mice by MOMA-2 staining (Fig. 3P). Over time, we did not observe any significant differences in the number of MOMA-2$^+$ cells in neither control nor diabetic plaques. Macrophage content, however, was higher in diabetic plaques at 8 and 15 weeks compared to control plaques. Comparing similar sized plaques from diabetic mice at 8 weeks and control mice at 15 weeks, no difference in the number of MOMA-2$^+$ cells was detected.

3.6. Differentiation of MNC towards SMLC in vitro is increased in diabetic apoE$^{-/-}$ mice

We previously described *in vitro* differentiation towards α-SMA$^+$ SMLC from spleen-derived MNC in DMEM culture medium supplemented with 20% serum [21]. Under these conditions, a proportion of spleen-derived MNC of untransplanted apoE$^{-/-}$ mice became adherent to the culture dish, adopted an elongated morphology after 8 days and expressed α-SMA (Supp Fig. IIA–D). Interestingly, we detected α-SMA positivity by flow cytometry in a large fraction of native spleen-derived MNC, although α-SMA signal intensity was lower than in SMLC and vascular SMC (Supp Fig. IE).

In the present study, we compared the effect of adding PDGF-BB, TGF-β or both to the DMEM culture medium on differentiation towards SMLC. After 3 and 8 days of culture, cells were stained for α-SMA and calponin. Adherent cells in all conditions stained positive for α-SMA and calponin as determined by immunofluorescence (Supp Fig. IIIA–P). At 8 days, α-SMA and calponin staining was higher than at 3 days and most intense staining was seen in PDGF-BB + TGF-β conditions. Relative mRNA expression of α-SMA, calponin 1 and, caldesmon and SM-MHC was measured using semi-quantitative PCR (Supp Fig. IV). After 3 days of culture, only α-SMA and caldesmon were upregulated compared to pre-culture apoE$^{-/-}$ spleen MNC. After eight days of culture we observed major upregulation of all 4 genes, reaching the highest levels with the combination of PDGF-BB and TGF-β.

We next cultured spleen-derived MNC for 8 days in DMEM + PDGF-BB from control and diabetic GFP$_{BM}$→apoE$^{-/-}$ mice at 4 and 11 week time points. Numbers of α-SMA$^+$ SMLC were determined using flow cytometry. There was no significant difference over time in the number of α-SMA$^+$ SMLC in control mice (Supplemental Table III). In contrast, we observed a higher number of SMLC at 11 weeks compared to 4 weeks after diabetes induction. The number of SMLC cultured from diabetic mice was lower compared to control mice at 4 weeks but significantly higher at 11 weeks after diabetes induction. At all time points in both groups, the percentage of cultured spleen cells expressing GFP was high (>70%), indicating that the majority of cultured α-SMA$^+$ SMLC were BM-derived.

3.7. No differences in plasma SDF-1 levels of atherosclerotic mice

The chemokine SDF-1α is essential in stem cell mobilization, homing and recruitment of smooth muscle progenitor cells to neointimal hyperplasia [22]. Therefore, the levels of SDF-1α were determined in peripheral blood of control and diabetic GFP$_{BM}$→apoE$^{-/-}$ mice. We did not observe differences in plasma SDF-1α levels between diabetic and control mice at any time point (at 4 weeks 1.26 ± 0.16 ng/ml in diabetic mice vs 1.09 ± 0.27 ng/ml in controls; at 11 weeks 1.34 ± 0.10 ng/ml in diabetic vs 1.95 ± 0.28 ng/ml in control mice).

4. Discussion

Vascular SMC play an important role in atherosclerotic plaque development and progression, which is accelerated in diabetes. Our data show that in a model of early stage atherosclerosis development, a small amount of BM-derived SMLC is present in atherosclerotic plaques. Moreover, we report for the first time that diabetes not only increases the number of SMLC obtained in *in vitro* culture from circulating MNC, but also enhances the amount of BM-derived SMLC in the atherosclerotic plaque, which may contribute to the accelerated atherosclerosis in diabetes.

The origin of neointimal SMC in atherosclerosis has been under debate. Previously, the contribution of BM-derived SMC to neointima formation in animal models of transplant arteriosclerosis and mechanical vascular injury has been reported [5–7,12,13]. Fewer studies investigating the role of BM-derived SMC in atherosclerosis have been published. In one study, BM-derived α-SMA$^+$ SMC were reported in atherosclerotic plaques of apoE$^{-/-}$ mice fed a high fat diet for 8 weeks [6]. Similarly, Iwata et al. recently demonstrated the presence of BM-derived α-SMA$^+$ SMLC in a BM transplant model in apoE$^{-/-}$ mice on a high fat diet for 8 weeks [12]. In an elegant study by Yu et al., genetic lineage tracing revealed that, although BM-derived SMC-like cells are infrequent in advanced primary atherosclerotic plaques and absent in fibrous caps, these cells may secrete proinflammatory cytokines and mitogens that promote local SMC proliferation and plaque growth [14]. On the other hand, Bentzon and co-workers showed that, in atherosclerotic plaques of apoE$^{-/-}$ mice that received BM from sex-mismatched GFP$^+$ apoE$^{-/-}$ mice, not one single GFP$^+$ α-SMA$^+$ cell was observed at 20 or 32 weeks of age [10].

We examined the contribution of BM-derived cells to plaque development in irradiated apoE$^{-/-}$ mice transplanted with GFP$^+$ BM on a normal chow diet which allowed investigation of the origin of SMC found in early stage fibrous plaques. In our model, induction of diabetes by STZ administration caused markedly accelerated atherosclerosis over time, with significant increases in plaque size in diabetic GFP$_{BM}$→apoE$^{-/-}$ mice compared to control GFP$_{BM}$→apoE$^{-/-}$ mice. Importantly, we show for the first time that the influx of BM-derived GFP$^+$ cells over time is increased in atherosclerotic plaques from diabetic GFP$_{BM}$→apoE$^{-/-}$ mice compared to control GFP$_{BM}$→apoE$^{-/-}$ mice. When we compare plaques of similar size from diabetic apoE$^{-/-}$ mice at 8 weeks and from control apoE$^{-/-}$ mice at 15 weeks, higher numbers of GFP$^+$ BM-derived cells are present in the diabetic atherosclerotic lesions. Interestingly, this was associated with a higher number of α-SMA$^+$ cells but similar numbers of macrophages. Immunofluorescent stainings further revealed that there was a significant increase in the number of GFP$^+$ α-SMA$^+$ cells in diabetic plaques. In addition, a higher fraction of spleen-derived MNC differentiated *in vitro*

towards α-SMA$^+$ SMLC in diabetic mice compared to control mice. This latter observation is consistent with our previous human data [20,21]. The increased amount of α-SMA$^+$ cells in diabetic atherosclerotic plaques may (in part) be due to paracrine effects of BM-derived SMLC by releasing cytokines and growth factors which favor local SMC proliferation. Of note, injection of smooth muscle progenitor cells obtained from healthy humans into apoE$^{-/-}$ mice can promote changes in plaque composition towards a more stable phenotype, both during the development and the progression of atherosclerosis [23]. This suggests suggest that circulating progenitor cells have the potential to modulate atherosclerotic plaque development which in a diabetic setting may result in acceleration of disease through phenotypic changes in progenitor cells.

Our study does not allow definitive conclusions on the mechanism by which diabetes enhances the influx of BM-derived SMLC in the vascular wall. Previously, it has been reported that SDF-1α plays an important role in neointima formation after vascular injury in apoE$^{-/-}$ mice by regulating neointimal SMC content [22]. A transient increase in plasma SDF-1α levels was observed after vascular injury which was associated with an SDF-1α–dependent recruitment of circulating smooth muscle progenitor cells. We did not observe any differences in SDF-1α plasma levels between control and diabetic apoE$^{-/-}$ mice at any of the time points. However, the activity of dipeptidyl peptidase-4, for which SDF-1α is a substrate, was reported to be increased in diabetes [24] and could therefore have decreased the amount of plasma SDF-1α detectable by ELISA. Therefore, we cannot exclude the possible effect of SDF-1α on (BM-derived) SMLC mobilization and accumulation in diabetic atherosclerotic plaques.

Likewise, circulating or local factors may influence the differentiation of circulating MNC that enter the plaque. TGF-β was shown to play a role in the transdifferentiation of vascular progenitor cells towards α-SMA$^+$ SMLC [18,25]. Under the stimulus of TGF-β or PDGF-BB endothelial colony forming cells can transdifferentiate into an SMC-like phenotype, both *in vitro* as well as in vivo [26,27], and TGF-β promotes outgrowth of SMLC from CD14$^+$CD105$^+$ MNC *in vitro* [17]. We have not measured circulating or vascular TGF-β levels in the present study. Interestingly though, circulating levels of TGF-β were shown to be increased in diabetes [28] and TGF-β levels were elevated in the aorta of diabetic apoE$^{-/-}$ mice [29], suggesting that circulating vascular progenitors may encounter these cytokines in the circulation or in the vascular wall. In addition, we have previously reported increased expression of TGF-β and decreased expression of BMP-6 in diabetic murine cultured smooth muscle progenitor cells [21]. Such TGF-β/BMP-6 imbalance may be of consequence for the intimal lesions to which progenitor cells home, both affecting transdifferentiation of progenitor cells towards an SMLC phenotype, as well as affecting resident cells by delivering TGF-β.

4.1. Study limitations

Our study employed lethal irradiation and reconstitution of apoE$^{-/-}$ BM with BM from GFP mice that have the normal apoE genotype, which has been shown to have suppressive effects on neointima formation in apoE$^{-/-}$ mice [30]. In addition, we used α-SMA to identify SMC as it is considered a sensitive marker for SMC. However, α-SMA is not a definitive SMC lineage marker and can be expressed by other cell types. Although we confirmed the presence of α-SMA- and calponin-positive cells by immunofluorescence in atherosclerotic plaques in a subset of tissue sections, a limitation of our study is that we did not further characterize and quantify SMC using specific markers such as SM-MHC, SM22a, and smoothelin.

5. Conclusion

Understanding the origin of SMC in diabetic atherosclerosis development is necessary for improving therapeutic strategies to control recruitment and accumulation of SMC in plaques. Furthermore, in the era of stem cell therapy, elucidating the effects of diabetes on BM-derived cell differentiation and recruitment in vascular disease may enhance its potential in a therapeutic setting. Therapies aimed at enhancing vascular progenitor cell availability in patients with ischemic cardiovascular disease are currently receiving a lot of attention and various clinical trials have been initiated. In patients with diabetes such therapeutic strategies may stimulate pro-atherogenic and pro-fibrotic processes due to altered SMLC function.

In conclusion, we provide evidence that BM-derived SMLC may be involved in early, rapid atherosclerosis development in diabetes which is associated with increased circulating and plaque SMLC. Future studies investigating the mechanisms underlying recruitment, differentiation and paracrine activity of BM-derived cells under diabetic conditions will further elucidate the involvement of BM-derived cells in diabetic vascular disease and the consequence this may have on the potential of stem cell therapy in patients with diabetes.

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Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2012.11.017.

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